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Phosphorylation of A170 Stress Protein by Casein Kinase II-like Activity in Macrophages

Toru Yanagawa,* Koichi Yuki,* Hiroshi Yoshida,† Shiro Bannai,* and Tetsuro Ishii*,[1]

**Department of Biochemistry, Institute of Basic Medical Sciences, and †Department of Oral and Maxillofacial Surgery, Institute of Clinical Sciences, University of Tsukuba, Tsukuba, Ibaraki 305, Japan*

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A170 is an oxidative stress-inducible protein having a Zinc finger domain, two PEST sequences, and many potential phosphorylation sites for serine/threonine kinases. These structural features suggest that the phosphorylation of A170 affects its function and degradation. We have found that A170 is phosphorylated in cultured murine peritoneal macrophages. In addition, using recombinant A170 proteins, we found two proteins of 40 and 44 kDa with kinase activity in cell extracts using an in-gel kinase assay. We compared the properties of the intrinsic A170 kinases with those of mitogen-activated protein kinase (ERK 2), protein kinase A (PKA), casein kinase II (CK II), and protein kinase C, since their catalytic subunits have molecular masses similar to A170 kinases. ERK 2, CK II, and PKA phosphorylated recombinant A170 as a substrate. The 40 and 44 kDa kinases present in the macrophage extract were similar to α and α' subunits of CK II in respect to substrate specificity, pharmacological properties, immuno-reactivities, and ubiquitous expression in tissues.

A170 is an oxidative stress-inducible protein found in murine macrophages (1,2). It has two PEST sequences with high scores and many potential phosphorylation sites for serine/threonine kinases (1). Based on the putative phosphorylation sites we hypothesize that the role of A170 is in signal transduction pathways triggered by various stresses. The importance of stress protein phosphorylation has been highlighted by recent discoveries that stress signals activate distinct protein kinases (3). and that phosphatase activity, such as that of CL100, is regulated by oxidative stress (4).

[1] To whom correspondence should be addressed at Department of Biochemistry, Institute of Basic Medical Sciences, University of Tsukuba, 1-1-1 Tennodai, Tsukuba-shi, Ibaraki-ken, 305 Japan. Fax: 81-298-53-3061. E-mail: teishii@md.tsukuba.ac.jp.

Furthermore, since A170 contains PEST sequences (5), it is assumed to have a short half-life in cells. We have previously shown that the expression of A170 is regulated at both the mRNA and post-transcriptional levels (2), and that p62, which is homologous to A170, affects signal transduction partly through ubiquitination-mediated protein degradation (6). Regulation of the stability of Fos, I kappa B (IκB) that have PEST sequences are reported to be under control of protein kinase phosphorylations by mitogen-activated protein kinase (MAPK), casein kinase II (CK II)(7,8). Therefore, it is important to identify the protein kinase that phosphorylates A170 in order to determine the mechanism of induction of stress proteins.

The structure of A170 has a high degree of homology with p62, a phosphotyrosine-independent ligand of the SH2 domain of the tyrosine kinase p56^{lck} (9); EBI3-associated protein, associated with a novel hematopoietin receptor induced in B-lymphocytes by Epstein-Barr virus infection (10); and ZIP, a protein kinase C (PKC)-ζ binding protein (11). Thus, because A170-related proteins are implicated in signal transduction pathways that regulate differentiation, proliferation or apoptosis, we hypothesize that A170 plays a significant role in signal transduction pathways.

In this study we examined protein kinases that phosphorylate A170 using an in-gel kinase assay. We found two protein kinase bands that phosphorylated the recombinant A170 embedded in SDS-polyacrylamide gel. The kinases differ from PKC-ζ that phosphorylated ZIP (11), so we characterized these kinases and found them to be similar to the α and α' catalytic subunits of CK II.

MATERIALS AND METHODS

Culture of cells and preparation of the cell extracts. Peritoneal macrophages obtained from ddY female mice were cultured in RPMI1640 medium containing 10% fetal bovine serum, as described previously (1). J774, a murine monocyte/macrophage cell line, and C6, a rat astroglioma cell line (ATCC CCL 107) obtained from Flow Laboratory were cultured in the same medium as above. Each stress

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agent was added to the culture medium 1 h after seeding macrophages, and cultured for the time periods indicated in the figure legends. After washing with PBS, whole-cell extracts were prepared by adding ice-cold lysis buffer (50 mM Tris, 150 mM NaCl, 2.5 mM $MgCl_2$, 10 mM EDTA, 0.5% TritonX-100, 1 mM Na_3VO_4, 10 μg of leupeptin per ml and 10 μg of *p*-amidino phenyl methanesulfonyl fluoride hydrochloride per ml) followed by centrifugation of the extracts at 12,000 rpm for 15 min. Tissues were excised from mice, washed with PBS, homogenized in SDS sample buffer and prepared in the same manner as whole cell extracts of macrophages. Protein concentrations were determined with the BCA protein assay reagent (Piece).

Preparation of recombinant A170 proteins. Three types of recombinant A170 proteins were obtained as shown in Fig. 1A. The N terminal portion of A170 (r-A170N), reverse N terminal sequence of A170 (r-A170NR), and the full length of A170 (r-A170) were obtained using the pET-23c expression vector system (Novagen). The oligonucleotide encoding the N terminus of A170, including amino acids 1 to 88, was amplified by PCR with oligonucleotide primers: 5′-GGACATAGCCATTGTCAGCT-3′ and 5′-ATGGCGTCGTTCACGGTG-3′. TA cloning was then performed and fused epitope tags in frame with their N-terminal (FLAG tag) and C-terminal (HIS tag) coding sequences (pET-23c). The antisense sequence of the N terminus of A170 was synthesized as a control. The reverse sequence encodes a protein that has no consensus phosphorylation site but has the same molecular mass as r-A170N. To obtain the full length of A170 protein, the original A170 cDNA sequence previously described (1) was fused to r-A170N cDNAs after digestion with Eco52 and XhoI. Recombinant proteins were produced using BL21 (DE3) competent cells and purified with His·Bind metal chelation resin (Novagen).

Reagents. Recombinant MAP kinase (ERK 2) was purchased from New England Bio Labs, Inc. The α catalytic subunit of Protein kinase A (PKA) (murine, recombinant) was from BIOMOL research Labs, Inc. Casein kinase II was from Promega. Protein kinase C (PKC), a mixture of α, β and γ isoforms, was from Seikagaku Corporation Japan. Paraquat and α-casein were from Wako Pure Chemical Industries Japan. Recombinant murine Tumor Necrosis Factor-α (TNF-α) was from PeproTech Inc. Epidermal growth factor was from Mallinckrodt. Lipopolysaccharide was from Difco. Heparin, with an average molecular weight of 3000, and myelin basic protein (MBP) were from Sigma.

Metabolic phosphorylation of A170 protein in the macrophages. Murine peritoneal macrophages were first cultured for 4 h at 5×10^7 cells per 10 ml of phosphate-free RPMI 1640 (Gibco) with 10% dialyzed fetal bovine serum in a 90 mm diameter dish, and 10 mCi of ^{32}P (PHOSPHORUS-32: Amersham) was then added to the medium before incubation for another 3 h at 37°C. After washing with PBS, whole-cell extracts were prepared by adding ice-cold lysis buffer. This extract was then incubated with rabbit polyclonal anti-A170 serum raised against recombinant A170 (1) for 2 h and immunoprecipitated with protein A Sepharose (Pharmacia Biotech). Precipitated protein was separated on a 10% polyacrylamide-SDS-gel, dried onto 3MM paper, and autoradiographed with Hyperfilm MP (Amersham).

In-gel kinase assay and in vitro phosphorylation. Various amounts of total cellular protein or kinases, as indicated in each figure, were resolved on a 10% polyacrylamide-SDS-gel containing 0.3 mg/ml of r-A170 recombinant proteins. The in-gel kinase assay was performed as originally described by Kameshita et al. (12). The gels were phosphorylated with 25 μM ATP, 25 μCi of [γ^{32}P] ATP at 25°C for 1 h. After washing, the gels were dried and autoradiographed with Hyperfilm MP or by BAS 5000 bioimaging analyzer (Fujifilm Japan).

To confirm that the bands were not autophosphorylated, portions of each phosphorylated band were excised, homogenized and extracted with buffer containing 50 mM NH_4HCO_3, 10% 2-mercaptoethanol, and 1% SDS. The protein samples were then electrophoresed, dried and autoradiographed.

In vitro kinase assay for intrinsic kinase in the macrophage extract and PKC were performed as follows. For intrinsic kinase assays, protein A Sepharose beads conjugated with or without r-A170 protein (10 μg) by T7 monoclonal antibody (Novagen, 5 μg) were incubated with 100 μg protein of the macrophage extract containing 50 mM Tris-HCl [pH 7.5] 0.5% Triton X-100, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 200 μM ATP and 10 μCi of [γ^{32}P] ATP at 37°C for 15 min. After incubation, r-A170 conjugated beads were washed three times, electrophoresed on SDS-PAGE and autoradiographed. For PKC, r-A170 and 10 μg of MBP as a control were incubated with one unit of PKC at 30°C for 30 min in reaction buffers containing 25 mM Tris-HCl (pH 7.0), 5 mM $MgCl_2$, 50 μg/ml phosphatidyl serine, 1 mM $CaCl_2$, 400 μM ATP and 10μCi of [γ^{32}P] ATP; but 1 mM EGTA was added instead of the 50 μg/ml phosphatidyl serine and 1 mM $CaCl_2$ in the controls.

Immunological analysis. Immunodepletion of CK II from the macrophage extract protein was performed as described in (13): cellular extract (50μg) was subjected to three rounds of immunodepletion using two types of anti-CK II antibodies (15μg): anti-CK II α mouse monoclonal antibody (Boehringer Mannheim GmbH), and anti-CK II α, α' rabbit polyclonal antibody (Upstream Biotechnology, Inc.). Each round included 6 h incubation with 5μg of antibody or non-immune serum at 4°C and then a 1 h incubation with protein G agarose (GIBCO BRL) or protein A Sepharose at 4°C. Equal amounts of each immunodepleted supernatant were analyzed by in-gel assay.

Immunoblotting was performed according to the instructions of the Amersham ECL system. Samples and positive control (EGF-stimulated A431 cell extract: Upstream Biotechnology, Inc.) were electrophoresed on SDS-PAGE and transferred onto nitrocellulose membranes. The membranes were blocked and incubated with anti-CK II α, α' rabbit polyclonal antibody (2μg/ml) in PBS containing 3% skim milk at 37°C for 1 h. After extensive washing, membranes were incubated with polyclonal donkey anti-rabbit IgG antibody (1:10000, v/v) conjugated with horseradish peroxidase (Amersham). The membranes were then washed in PBS, followed by detection with the ECL system and Hyperfilm ECL (Amersham).

RESULTS

A170 was phosphorylated in murine peritoneal macrophages. Since A170 had many putative phosphorylation sites, we determined whether A170 was phosphorylated in murine peritoneal macrophages cultured *in vitro*. The macrophages were metabolically labeled with ^{32}P Phosphorus and cellular proteins were immunoprecipitated with anti-A170 serum as described in the experimental procedures. A single protein band of 60 kDa corresponding to cellular A170 was observed in the immunoprecipitates (Fig. 1B, Lane 1) indicating that A170 was phosphorylated in the cells. To determine whether or not the kinase(s) in macrophage extracts phosphorylate recombinant A170 as a substrate, beads tagged with r-A170 were reacted with the extract. The results indicate that kinase(s) in macrophage extracts phosphorylate r-A170 as a substrate (Fig. 1B, Lane 2,3).

Detection of cellular kinases that phosphorylate A170. An SDS-PAGE-based in-gel phosphorylation assay was used to detect the cellular protein kinases

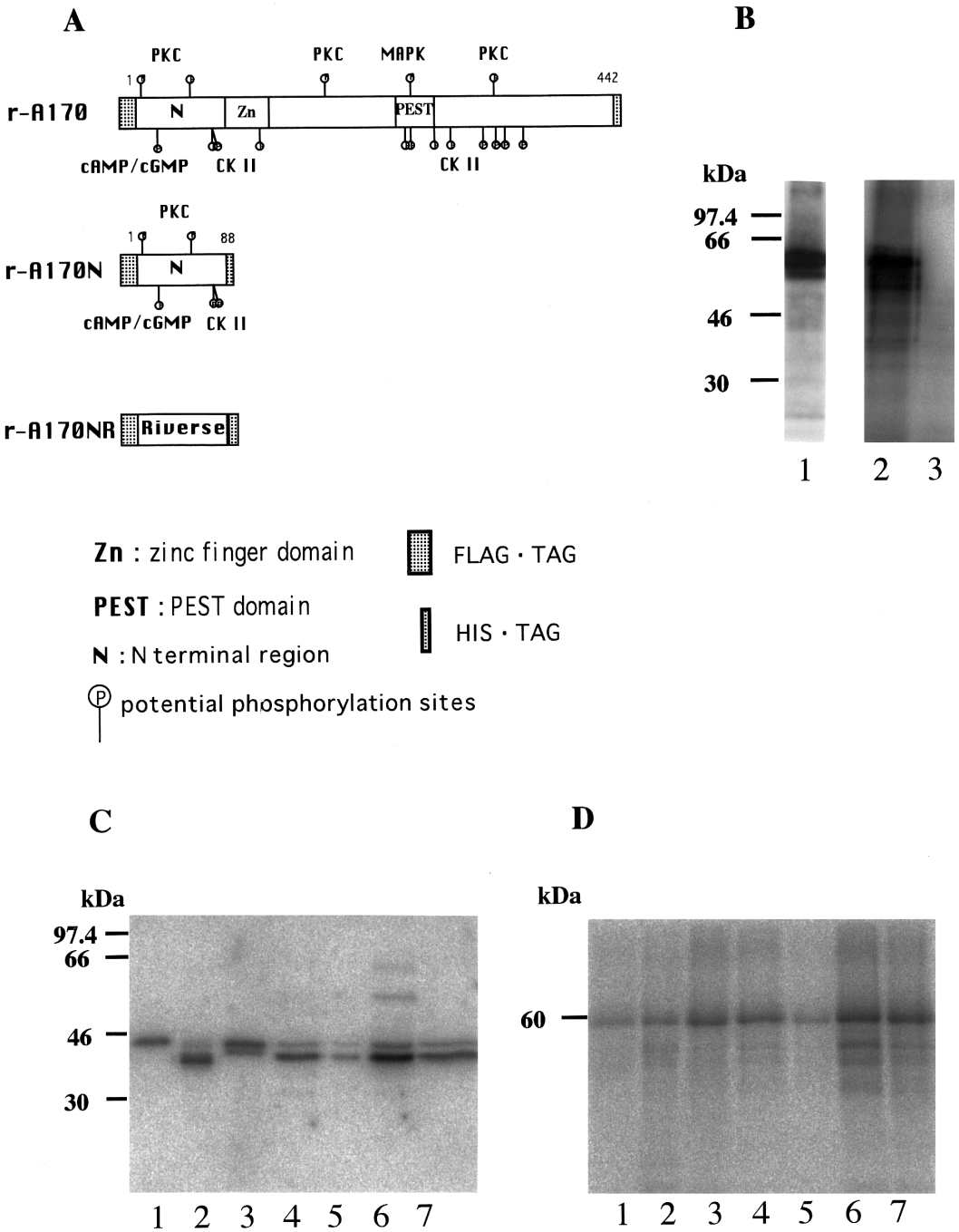

FIG. 1. Phosphorylation of A170 protein. (A) Schematic representation of the A170 fusion proteins. Potential phosphorylation sites of casein kinase II (CK II), MAP kinase (MAPK), protein kinase C (PKC), and cAMP/cGMP dependent protein kinase (cAMP/cGMP) are shown, respectively. (B) Metabolic labeling of the A170 protein with [^{32}P] Phosphorus. The macrophages were labeled with [^{32}P] and the extract was immunoprecipitated with anti-A170 polyclonal serum. Lane 1 shows ^{32}P-labeled A170 protein detected by autoradiography. Lane 2 shows in vitro phosphorylation of beads conjugated with r-A170 macrophage extract. Lane 3 is a control of lane 2 without r-A170. (C) In-gel kinase assay using the recombinant A170 protein as the substrate. Lane 1, ERK 2 (500U); lane 2, CK II (10U); lane 3, PKA (0.5U); lane 4, macrophage extract (20μg protein); lane 5, Macrophage extract (5μg); lane 6, J774 cell extract (20μg); lane 7, C6 cell extract (20μg). (D) Electrophoresis of the extracted ^{32}P-labeled bands in C between 30 and 50 kDa in SDS-PAGE. Lane 1, ERK 2; lane 2, CK II; lane 3, PKA; lane 4, macrophage extract 40 kDa band; lane 5, macrophage extract 44 kDa band; lane 6, J774 cell extract; lane 7, C6 cell.

that phosphorylate A170. Cell extracts (20 μg protein) from paraquat-treated (100 μM, 7 h) murine peritoneal macrophages were separated in the acrylamide gels containing recombinant A170 proteins and the kinase assay was performed. In the macrophage extracts we detected two major kinase activities of 40 and 44 kDa that phosphorylated r-A170 as a substrate (Fig. 1C, Lane 4, 5). To identify these intrinsic kinases, we compared the properties of the kinases found in the macrophage extract with those of purified ERK 2 (as MAPK), CK II, and PKA (as cAMP and cGMP-dependent kinases). Except for PKC, the apparent molecular masses of the purified kinases or their subunits are between 35 and 45 kDa. ERK 2 appeared as a 44 kDa band, rat CK II α and α' catalytic subunits appeared as 37 and 44 kDa bands, and PKA appeared as 42 and 44 kDa bands in respectively phosphorylated r-A170 (Fig. 1C, Lanes 1-3). We also detected expression of kinase in mouse macrophage cell line J774 and rat astroglioma cell line C6 (Fig. 1C, Lane 6,7) suggesting ubiquitous expression of the kinase activities. These kinases at the indicated doses phosphorylated A170 to a similar extent as shown in Fig. 1C. To confirm that these bands were not autophosphorylated kinases, but were derived from phosphorylated 60 kDa A170 protein, the gel corresponding to each band was excised, eluted, and separated by SDS-PAGE. In each case, the major radioactive band was a 60 kDa, which is the size of the r-A170 recombinant protein (Fig. 1D). Two radioactive bands in the range of 40 to 44 kDa were detected in cell extracts using in-gel assays containing r-A170 and r-A170N. However, no kinase activity was detected in the gel polymerized with r-A170NR (data not shown), which suggests that the tagged? part was unrelated to this kinase activity. PKC has a mass of 80 kDa, and it did not phosphorylate A170 by in vitro kinase assay (data not shown). Therefore, PKC (α,β,γ) is not identical to the major kinases detected in the macrophage extracts.

To examine expression of the cellular kinases, major murine organs (brain, lung, heart, liver, pancreas, spleen, kidney, muscle, thymus, adrenal gland, ovary and uterus: 20 μg) were subjected to in-gel kinase assay. The 40 and 44 kDa kinase activities were ubiquitously expressed among the tissues (data not shown).

Substrate specificity of A170 kinase. To compare the substrate specificity of the cellular 40 and 44 kDa kinases with the other kinases, we performed in-gel assay using α-casein and MBP as the substrates. As shown in Fig. 2A and B, MBP is a good substrate for MAPK and PKA, while α-casein is the substrate for CK II, MAPK, and PKA. Three authentic kinases and the cell extracts phosphorylated α-casein embedded in the gel (Fig. 2A), but the cell extract and CK II did not phosphorylate MBP (Fig. 2B). These results show that cellular A170 kinase and CK II have similar substrate specificity.

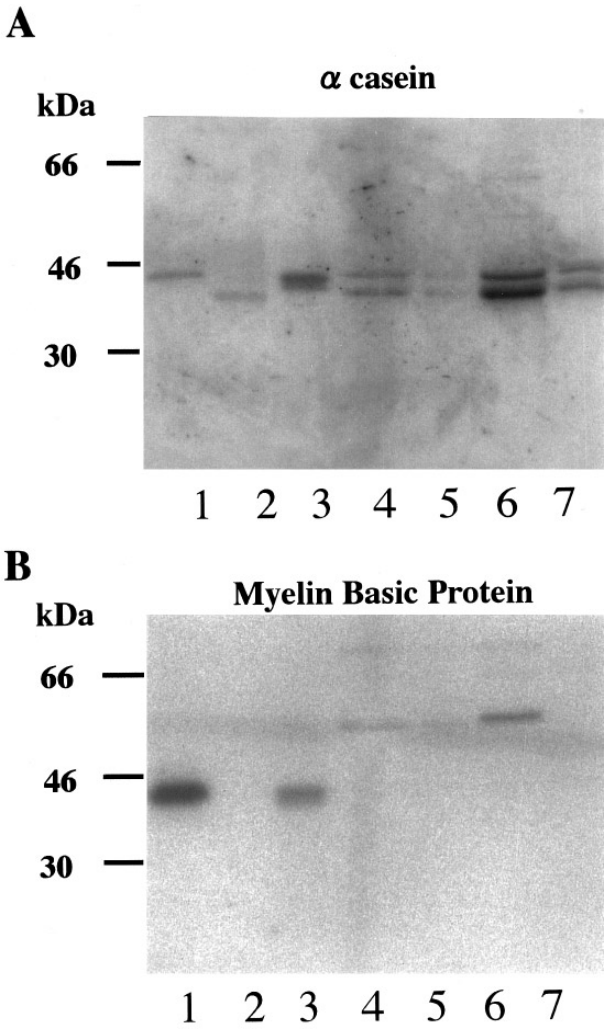

FIG. 2. Substrate-specificity of the kinases. (A) 0.3 mg/ml of α-casein and (B) 0.3 mg/ml of myelin basic protein (MBP) were polymerized in separate SDS-PAGE gels and in-gel kinase assays were performed. Lane 1, ERK 2 (500 U); lane 2, CK II (10 U); lane 3, PKA (0.5 U); lane 4, macrophage extract (20 μg); lane 5, macrophage extract (5 μg); lane 6, J774 cell extract (20 μg); lane 7, C6 cell extract (20 μg).

Pharmacological property of A170 kinase and immuno-analysis of A170 kinase by casein kinase II antibody. CK II has the characteristic properties of being inhibited by heparin (14) and being able to utilize GTP instead of ATP (15). We therefore used in-gel assays to determine whether A170 kinase was inhibited by 50 nM of heparin and 1 mM of GTP. Phosphorylation by A170 kinase and CK II was inhibited by both treatments, but PKA was not inhibited under similar conditions (Fig. 3A).

To determine whether or not the 40 and 44 kDa kinases were cross reactive with anti-CK II antibody, we

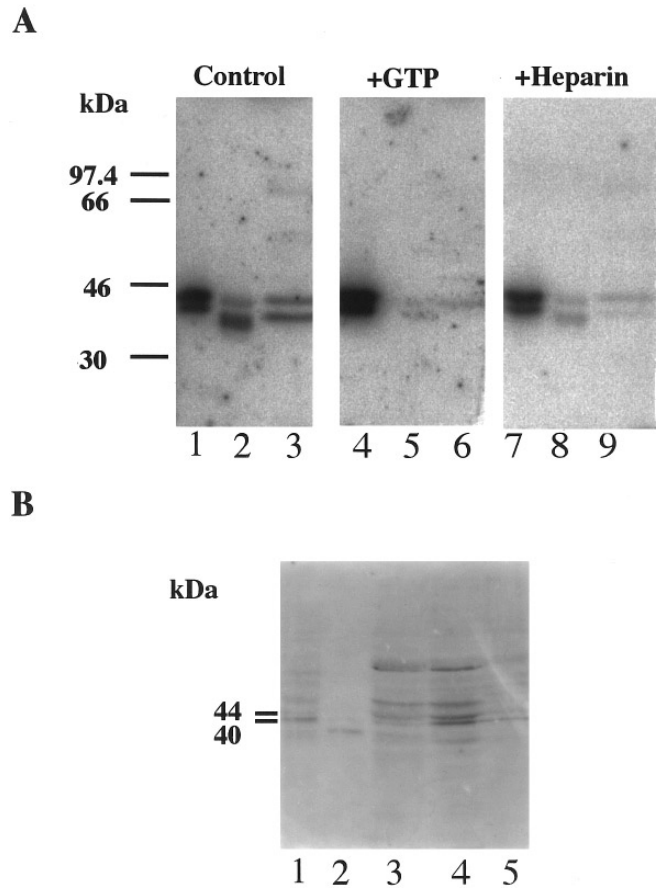

FIG. 3. Pharmacological and immunochemical properties of A170 kinase. (A) Pharmacological property of A170 kinase. PKA (0.5U), CK II (10U), and macrophage extract (20 μg) were electrophoresed on SDS-PAGE containing r-A170. Lanes 1 to 3, control: PKA (lane 1), CK II (lane 2), A170 kinase (lane 3). Lanes 4-6, phosphorylation in the presence of 1 mM GTP: PKA (lane 4), CK II (lane 5), A170 kinase (lane 6). Lanes 7-9, phosphorylated in the presence of 50 nM heparin: PKA (lane 7), CK II (lane 8), A170 kinase (lane 9). (B) Immunoblot analysis with anti-CK II antibody. EGF-stimulated A431 cell extract (lane 1), CK II (10U, lane 2), macrophage extract (50 μg, lane 3), J774 cell (50 μg, lane 4), and C6 cell (50 μg, lane 5).

tested immunodepletion of the kinase activity with two kinds of anti-CK II antibody, but these antibodies did not deplete the activities of the kinase effectively under these conditions (data not shown).

Immunoblot analysis showed that there were immunoreactive bands corresponding to the CK II α and α' subunits in the EGF-stimulated A431 cell extract (positive control), purified rat liver CK II, macrophage cell extract, J774 cell extract and C6 cell extract within the range of existing A170 kinase activities (Fig. 3B), so it appears that the activities of 40 and 44 kDa kinases correspond to the α and α' subunits of CK II.

Effect of treatment with various agents. Since A170 is an oxidative stress-inducible protein (2), we monitored the levels of cellular kinases during treatment with paraquat. In-gel assays were performed with cell extracts that had been incubated for 15 min, 30 min, 3 h, 5 h and 7 h with and without paraquat. The apparent kinase activities were slightly elevated after 1-7 h of incubation, and were hardly affected by paraquat (data not shown).

We next examined the effects of the other agents that affected activities of MAP kinase families. Macrophages were incubated with TNF-α (30 nM), LPS (10 nM) or EGF (10 nM) for 15 min or 30 min. MAPK and Jun N-terminal kinase (JNK) activities were elevated around 15 to 30 min as described previously (16), but these kinase activities did not respond to this stimulation (data not shown).

DISCUSSION

We have shown in this report that MAPK (ERK 2), PKA and CK II phosphorylate A170 recombinant protein *in vitro*. Also, using the in-gel assay we detected protein kinases of 40 and 44 kDa as the major macrophage kinases that phosphorylate A170 protein. All of the results presented here (molecular size, substrate specificity, inhibition of phosphorylation by heparin and high concentration of GTP, and ubiquitous distribution of the two kinases) suggest that the two cellular kinases are quite similar to the α and α' catalytic subunits of CK II.

We tested immunodepletion of the kinase activities, but found no immunoprecipitation. This result is probably due to the fact that the affinity of the antibodies to CK II is not as high as shown in immunostaining (Fig. 3B), and that CK II exists bound to a variety of proteins, such as heat shock protein (HSP90), p53 and others (17,18).

CK II is a tetramer consisting of α, α', β and β subunits, with the β subunit having an inhibitory activity (19). The inhibitory subunits were separated from the catalytic subunits by in-gel assay. It is therefore necessary to detect the CK II kinase activity of the whole complex in the cell extract by using a CK II specific substrate to monitor possible changes in its activity. This idea can be also applied to PKA, which consists of catalytic subunits and regulatory subunits.

A170 was cloned as an oxidative stress-induced protein, in which low levels of oxidative stress induce its accumulation (1,2). The internal or external oxidative stress signals are transduced through various pathways, one of which is through the activation of tyrosine kinases. Lck is known as the tyrosine kinase which transduces signals. Although there are many reports about oxidative stress and Lck (20-24), there remain some disagreements regarding the relation between oxidative stresses and Lck activation in many experiments. Some modulators for oxidative signals could explain these findings because p62, which is homologous

to A170, binds to the Lck SH2 domain in a phosphotyrosine-independent manner, and A170 is induced by oxidative stress. So we can speculate that one function of A170 may be to modulate signal transduction in response to oxidative stress.

On the other hand, oxidative stress response is observed as heightened serine-threonine kinase activities. Oxidative stress induces CL100, a protein phosphatase modulating the activation of MAP kinase (4). JNK is responsive to H_2O_2 and transduces apoptosis signals (25). PKA activity depends on cAMP concentration, and oxidant stress enhances adenylyl cyclase activation (26). Because A170 has many potential phosphorylation sites, these kinases can transduce oxidative stress signals through phosphorylation of A170. In this study, responses were observed when various stimulants were added. TNF-α was chosen as the stimulant for JNK, EGF for MAPK, and LPS for p38 MAPK. Paraquat was used as the control oxidative stress agent. The intrinsic 40 and 44 kDa kinase activities were not transiently elevated by these stimulants. Recently, IκB-α kinase, which has CK II-like properties, was identified and its kinase activity was found to be rapidly elevated by TNF-α stimulation (16). We therefore treated macrophages with TNF-α (300 unit/ml) for 1 min and 5 min and determined the kinase activities by in-gel assay, but could not detect any change (data not shown). These results consistently show that A170 kinase is neither an MAPK family kinase nor an IκB-α kinase.

PKC (a mixture of α, β and γ) did not phosphorylate the recombinant A170, which result was in good agreement with ZIP. Recently, ZIP that has an amino acid sequence with 97% identity to A170 was cloned as the binding protein to PKC-ζ. Although it was phosphorylated with PKC-ζ, it was not phosphorylated with PKC-α, β or γ (11). As shown in this report, A170 kinase is 40 to 44 kDa. Moreover, although the TNF signal is transmitted through the MAPK pathway to PKC-ζ (27), A170 kinase did not respond to TNF stimulation. Therefore the kinase activities apparently do not involve PKC-ζ.

The interaction of ZIP with PCK-ζ, or ZIP with ZIP, alters the subcellular localization of ZIP, but the kinase activity of PKC-ζ is independent from this interaction (11). On the other hand, CK II is reported to regulate the interaction of proteins. Basal phosphorylation by CK II regulates functional interaction with proto-oncogene product (28), so it is possible that CK II plays a role in regulating the interaction of ZIP with PKC-ζ and in auto-association of ZIP.

In addition, CK II is responsive to viral infection and various stresses, and regulates IκB degradation (13). IκB has a PEST sequence and the importance of its constitutive phosphorylation by CK II has been indicated (29,30). A170 also has PEST sequences and is phosphorylated by CK II, so it is not unreasonable to assume that A170 is phosphorylated basically by CK II, and that its turnover is regulated by the phosphorylation of CK II.

In this study we detected one of the kinases that phosphorylated A170 and discuss where the kinase is placed in the pathway of signal transduction. The 40 and 44 kDa A170 kinase that mainly phosphorylates A170 differs from PCK-ζ, PKA and ERK 2, but it is likely to be CK II or a kinase very similar to CK II. More intensive studies of the kinetic activity of CK II and further analysis of the phosphorylation site of A170 are necessary to reveal the stress response mechanism and regulation of A170 so that we can better ascertain the role of A170 in the signal pathway and regulatory system.

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